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Tubulin, the dimeric protein of microtubules, exists as various isoforms in different tissues and species. Tubulin is the target protein for various antitumor drugs such as paclitaxel, vinblastine and vincristine, which are routinely used for cancer chemotherapy. Previous studies from this laboratory have shown that certain tubulin isoform exhibit preferential interaction with antitumor drugs. Thus, the isoform composition of a tissue may affect the antimitotic properties of any drug. Here, breast cancer cells were tested for the presence of different tubulin isoforms by immunoblotting and RT-PCR analysis. Breast cancer cells were made resistant to antitumor drugs paclitaxel and podophyllotoxin and the tubulin isoform content was studied. The results show that paclitaxel-resistant MCF-7 cells had 2-3 fold increase in β_{II} and β_{III} expression than that of the wild type. On the other hand, podophyllotoxin-resistant MDA-MB-231 cells had an increased expression of β_{VI} than that of the wild type cells. A full length cDNA for β_{III} was prepared. This cDNA will be used to construct a plasmid for the overexpression of β_{III} tubulin in the breast cancer cells. The cells will be tested for the sensitivity to antitumor drugs. These results will be very important for proper selection as well as design of novel drugs for breast cancer.

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A Banerjee 6/7/00

PI - Signature Date

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Introduction

Tubulin, the αβ dimeric protein of microtubules, is the target of various antitumor drugs such as paclitaxel, vinblastine, and vincristine. Both α– and β-tubulin occur as different isoforms which are expressed in a tissue-specific manner. Our earlier results have demonstrated that the antimitotic drugs colchicine and its analogs exhibit differential interaction with different β–tubulin isoforms. The primary goal of this project is to study the role played by individual tubulin isoforms in the drug sensitivity of breast cancer cells. There are about 5-6 α–tubulin and as many as 6-7 β–tubulin forms in human system. Efforts will be initiated to study the β–tubulin isoforms in the breast cancer cells. Furthermore, to test whether isoform composition can affect drug sensitivity, full length cDNA specific for different tubulin isoforms will be prepared and then will be inserted into breast cancer cells. The

Report Body

Preparation of drug-resistant Breast Cancer Cells:

It has been reported that certain tubulin isoforms get expressed when cancer cells get resistant to anticancer drugs. To study the tubulin isoforms efforts were initiated to prepare breast cancer cells resistant to antimitotic drugs. The cell lines were prepared by initially growing breast cancer cell lines MCF-7 and MDA-MB-231 in the presence of 1 nM of colchicine, podophyllotoxin, vinblastine or paclitaxel. Verapamil was kept in the growth medium to exclude multidrug-resistant cells. The drug concentration was gradually increased by 1.5 times. After 3-4 months of selection, two drug-resistant lines **MCF-7/PTX20** (resistant to paclitaxel) and **MDA-MB-231/POD60** (resistant to podophyllotoxin) were obtained.

Immunoblot analysis of β–tubulin isoforms in drug-resistant breast cancer cells:

The drug-resistant breast cancer cells were grown in suitable medium in T-150 culture flasks to confluency. The cells were trypsinized and harvested after washing twice in sterile PBS. The cell pellet s were homogenised in PBS using 1 ml glass homogeniser and homogenate was centrifuged at 20,000 rpm in a sorvall centrifuge for 1 h. The cell extract was mixed with equal volume of 2X Laemmli sample buffer, boiled for 5 min, and was analyzed by SDS-polyacrylamide gel electrophoresis and immunobloting using monoclonal antibodies to β_{II}, β_{III}, and β_{IV}.

As shown in figure 1, the paclitaxel-resistant MCF-7 cells contains much higher amounts of β_{II} and β_{III} as compared to the drug-sensitive wild type cells. The amount of β_{IV} was increased marginally in the resistant cells. On the other hand, podophyllotoxin-resistant cells exhibited a decrease in the content of all three isoforms β_{II}, β_{III}, and β_{IV}. Since no antibody was available, it was not possible to see the status of the other β–tubulin isoforms by immunoblotting.

RT-PCR studies:

To study the expression of different β–tubulin isoforms, toal RNA was isolated from breast cancer cells. The RNA was subjected to RT-PCR amplification and was visualized in agarose gels after staining with ethidium bromide.

β–tubulin-specific primers used for the polymerase chain reaction

CLASS	POSITION	SEQUENCE
I (HM40)	5'-UTR	Forward: 5'- ACCTCGCTGCTCCAGCCTCT-3' Reverse: 5'- CCGGCCTGGATGTGCACGAT-3'
II (Hβ9)	Coding	Forward: 5'- CGCATCTCCGAGCAGTTCAC-3' Reverse: 5'- TCGCCCTCCTCCTCCTCGA-3'
III (Hβ4)	3'-UTR	Forward: 5'- CTGCTCGCAGCTGGAGTGAG-3' Reverse: 5'- CATAAATACTGCAGGAGGGC-3'
IV a (Hβ5)	5'-UTR	Forward: 5'- TCTCCGCCGCATCTTCCACC-3' Reverse: 5'- CCGGCCTGGATGTGCACGAT-3'
IV b (Hβ2)	5'-UTR	Forward: 5'- GAGCTTGCCAGCCTCGTTCT-3' Reverse: 5'- CCGATCTGGTTGCCGCACTG-3'
VI (Hβ1)	5'-UTR	Forward: 5'- ACAGTGTGTTGGCTCACACC-3' Reverse: 5'- CCGATCTGGTTGCCGCACTG-3'
Human GAPDH		Forward: 5' GTT CGA CAG TCA GCC GCA TCT 3' Reverse: 5' GGC ATG GAC TGT GGT CAT GAG 3'

The expression of different β–tubulin isoforms was studied in MCF-7 cells as well as in paclitaxel resistant MCF-7/PTX20 cells by RT PCR amplification of total RNA. As shown in fig. 2, the expression of β_I, β_{II}, β_{III} and β_{VI} is increased significantly. On the other hand β_{IVa} and β_{IVb} is decreased.

At this point it is not clear why the level of some of the isoforms gets elevated while that of others decrease. It may be possible that cells can identify those isoforms that have the lowest interaction with the drug, and specifically overexpress those isoforms, while the isoforms that have the highest affinity for the drug get lower expression. In order to test this hypothesis, individual α–and β–tubulin

Preparation of full length cDNA for β_{III} tubulin

To overexpress individual tubulin isoforms it will be necessary to make full length cDNA specific for individual tubulin isoforms. By using primers specific for β_{III} tubulin we have prepared full length 1350 bp cDNA from total RNA isolated from MCF-7 cells by RT-PCR. The product shows a single 1350 bp band in 1.5% agarose gel. We are planning to construct a plasmid for the overexpression of this full length cDNA in breast cancer cells.

Key Research Accomplishments

- We have studied the expression of tubulin isoforms in breast cancer cell lines by immunoblotting and RT-PCR analysis.

- Paclitaxel resistant MCF-7/PTX20 cells express increased amounts of β_{II} , β_{III} but not β_{IV}.

- Podophyllotoxin resistant MDA-MB-231/POD60 cells express lower amounts of β_{II} , β_{III}, and β_{IV}.

- RT-PCR analysis of the total RNA from paclitaxel resistant cells show the increasd expression of β_{II}, and β_{III}, while an inhibition β_{IVb} is observed.

- We have prepared a full length cDNA for β_{III} from MCF-7 cells.

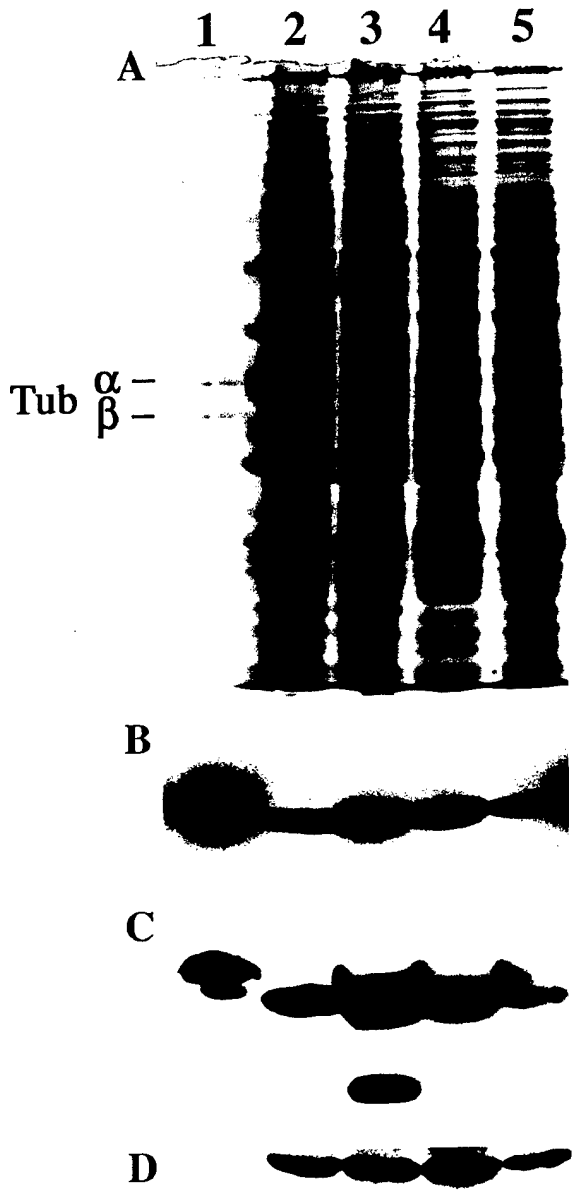
Figure Legends:

Figure 1. Immunoblotting analysis of the β–tubulin isoforms in the cell extracts from drug-resistant Breast cancer cells

The cell extracts were boiled with Laemmli sample buffer for 5 min and were subjected to SDS PAGE on 7.5 % polyacrylamide gel (Panel A). Three identical gels were transblotted onto nitrocellulose membranes for immunoblotting. The blots after the transfer were blocked with 5% milk and 0.1% BSA for 2h, incubated with antibodies specific for the β–tubulin isoforms for 1h and washed to remove the free antibody. The blots were subsequently incubated with horseradish peroxidase-coupled secondary antibody for 1 h. After washing off the secondary antibody, the blots were developed with an enhanced chemiluminescent HRP-substrate and exposed on Kodak XOmat X-ray films. The samples are: Lane 1, PC-tubulin from brain, lane 2: MCF-7 extract, lane 3, MCF-7/PTX20, lane 4, MDA-MB-231extract, lane 5, MDA-MB-231/POD60 extract. Loading in lanes 2-5 was 50 μg. Panel A: Gel; Panel B-D, Immunoblots. B, Anti-β_{II}; C, Anti-β_{III}, and D, Anti-β_{IV}. The region for α– and β– tubulin is indicated on the gel.

Figure 2. RT-PCR analysis of the β–tubulin isoforms in drug resistant breast cancer cells

Total RNA (1mg) from MCF-7 and MCF-7/PTX20 cells were first incubated with DNase I at room temperature for 10 min and then reverse transcribed with 2 units AMV reverse transcriptase for one hour in the presence of deoxyribonucleotides and oligo (dT) primers. An aliquot of each sample was amplified in the presence of Taq DNA polymerase for 32 cycles using primers specific for β–tubulin isoforms. The samples after amplification were loaded on a 1.5% agarose gel in tris-EDTA buffer. Ethidium bromide (0.5 μg/ml) was included in the running buffer for immidiate visualization of the DNA bands. Upper panel: Wild type MCF-7 cells; Lower panel: Paclitaxel resistant MCF-7/PTX20 cells. Individual β–tubulin isoforms are indicated by roman numerals, G stands for GAPDH.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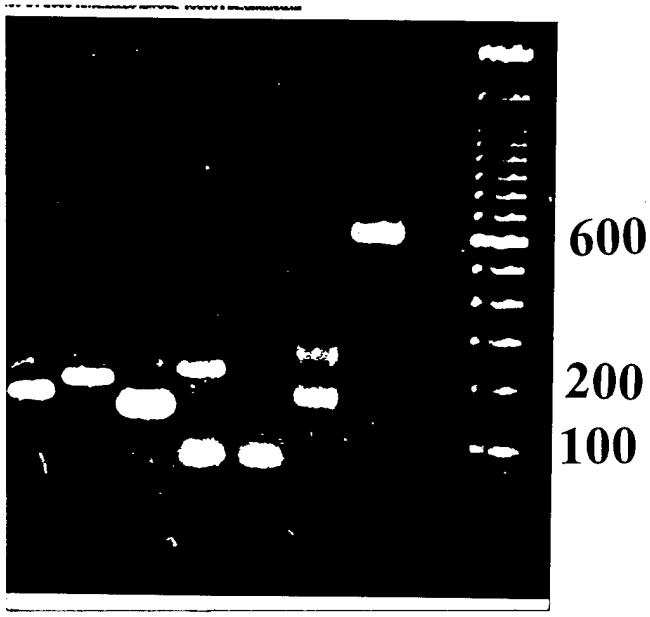

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